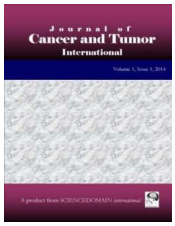

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Assessment of Kirsten Sarcoma Viral Gene Mutations in Non-neoplastic and Neoplastic Nodular Lesions of Thyroid in Pakistan

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Authors' contributions

This work was carried out in collaboration between all authors. Author FJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SIK managed the analyses of the study. Author KZA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: To assess KRAS mutations in non-neoplastic and neoplastic nodular lesions of thyroid as the incidence of KRAS mutations in thyroid lesions in Pakistan has not been evaluated.
Methods: The cross sectional study was conducted at Basic Medical Sciences Institute, Jinnah Postgraduate Medical Center, Karachi from 2011 to 2014. 70 cases including 6 multinodular goiters, 10 hyperplastic nodules, 10 follicular adenomas, 7 WDT-UMP, 4 follicular carcinomas, 22 classical papillary carcinomas, 11 follicular variant of papillary carcinomas were subjected to standard PCR to detect KRAS mutations located at codon 12 exon 1.
Results: KRAS mutations located at codon 12 exon 1 were found in 02(2.87%) cases of multinodular goiter, 05(7.14%) hyperplastic nodules, 02 (2.87%) follicular adenoma, 03(4.2%) WDT-UMP. Among malignant lesions follicular carcinoma showed KRAS positivity

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03(4.2%), classical papillary carcinoma 08(11.42%) and follicular variant of papillary carcinoma 07(10%).

Conclusion: Our data suggests strong presence of KRAS mutations in malignant tumors supporting the presence of KRAS mutations in our population particularly follicular variant of papillary carcinoma. Follicular variant is a discrete variant of papillary carcinoma having strong association with KRAS mutations.

Keywords: KRAS; FVPTC; RAS; WDT-UMP.

1. INTRODUCTION

Thyroid neoplasm represents most common endocrine malignancy with rising incidence [1]. It constitutes 90% of all endocrine cancers. Thyroid lesions most commonly present as thyroid nodules in 4-7% of population out of which 5-6.5% are malignant [2]. The thyroid cancer incidence has tripled, the reason could be increasing use of modern detection techniques and diagnostic criteria changes [3].

Worldwide thyroid cancer is the 16th most common cancer in 2012 [4]. In USA estimated prevalence of thyroid cancer in 2016 was 64,300 [5]. According to Shaukat Khanum cancer hospital and Research registry in 2013 thyroid carcinoma is the 10th most common malignancy in females with Papillary carcinoma being the most common histological type [6].

Possible risk factors for thyroid cancer are iodine deficiency or excess, radiation exposure and organo chlorinated pollutants [3,7-9]. Histologically thyroid neoplasms are classified as Papillary, Follicular, Medullary and Anaplastic tumors.

There has been substantial increase in understanding of molecular genetics of thyroid cancer over the past couple of years [10]. Various mutations are involved in thyroid neoplasms such as BRAF, RAS, RET/PTC and PAX/PPAR gamma rearrangements. RAS has been implicated as fundamental physiological and pathological process in controlling cell growth, differentiation and survival. RAS comprises of 150 distinct cellular members [11].

RAS accounts for second most common genetic alteration. RAS gene has 3 isoforms NRAS, HRAS, KRAS [1]. NRAS mutation at codon 61 is by far most common [10]. Thyroid neoplasms are unique as all of 3 isoforms of RAS are reported in thyroid neoplasms [12]. KRAS is the 6th most common genetic mutation in all cancers [3]. Higher percentages of KRAS mutations are reported in various organs. Incidence of KRAS mutations in pancreatic cancers are 61%, colonic cancers 33%, biliary tract cancers 31% and lung cancer 17% respectively [13]. Among thyroid cancers frequency of KRAS varies and is reported by Catalogue of somatic mutations in cancer (Cosmic) as NRAS 6%, HRAS 4%, KRAS 2 to 3% respectively [14]. The mutations in KRAS gene are mostly located at positions 12 and 13 in exon 1 and less in codon 61 close to the GTP binding site [15].

Studies have demonstrated that thyroid cancers bearing KRAS mutations have strong impact on tumor dedifferentiation, aggressiveness and distant metastasis [16]. There is a great discrepancy regarding overall frequency of KRAS mutations in follicular patterned lesions, some studies have reported incidence of KRAS mutations in colloid nodule 1%, adenoma 6% follicular carcinoma 1.6% and papillary carcinoma 2.7% [8]. The reason for this discrepancy may be the small sample sized studies and difference in techniques [1]. It was observed that KRAS mutations were seen more frequently in malignant than benign thyroid lesions [8]. The incidence of KRAS mutations in Pakistan has not been evaluated. This study was designed to detect KRAS mutations in neoplastic as well as non-neoplastic nodular thyroid lesions particularly in classical papillary carcinoma and its morphological variants as KRAS mutations are associated with tumor aggressiveness and poor outcome [3].

2. MATERIALS AND METHODS

Study approval was obtained from ethical committee of Basic Medical Sciences Institute, Jinnah Postgraduate Medical Center, Karachi with reference number (NO.F.1-2/2015/BMSI-E-COMT/022/JPMC) on 11 March 2015 and procedure was in accordance of Declaration of Helsinki. Properly fixed paraffin embedded surgical pathological specimens of patients who underwent thyroidectomy during 5 years (2011-2014) were retrieved from previous records. 70 cases were selected; these included 6 multinodular goiters, 10 hyperplastic nodules, 10

follicular adenomas, 7 WDT-UMP, 4 follicular carcinomas, 22 classical papillary carcinomas, 11 follicular variant of papillary carcinomas. Toxic multinodular goiter, metastatic carcinoma and non follicular thyroid neoplasm were excluded from study.

The DNA extraction from tissue was carried by using Epicenter Kit (cat#MCD 85201) and the protocol was followed accordingly. In 1.5 ml appendroff cup 50 mg of tissues was taken from paraffin block and 600 µl T and C lysis solution was added along with diluted 1 µl of 50 µg/µl Protinase K to all the sample tubes. Mix and incubate overnight at 37℃ then all the tubes were vortex and 20 µl of MPC (Protein Precipitation Reagent) was added to 600 µl of lysed sample and was vortexed vigorously. Samples were centrifuged at 13000 rpm for 10 minutes and supernatant was obtained. The supernatant was transferred into another tube in which 500 µl of isopropanol was already added. All the tubes were inverted 30-40 times slowly to recover the DNA from supernant. The tubes were centrifuged for 10 minutes at 13000 rpm. The supernatant was discarded without disturbing the pellet. Then 500 µl 100% ethanol was added into pellet and was centrifuged for 7 minutes at 13000 rpm to remove all the isopropanol. The supernant was discarded and tubes were placed inverted slowly without disturbing the pellet .The pellet was air dried and the pellet was resuspended in 35 µl of TBE buffer. All the samples were safed at -80℃ till PCR was performed.

Genotyping by standard PCR was performed in a tube containing 200 µl of reaction mixture made up of the following components: 20 pmol of each primer (forward and reverse), 500 µm of four deoxynucleotides, 2.5 U of Taq polymerase (Promega), 10 X PCR buffer containing and 1.5 mM $MgCl_2$. The thermal cycler (Master Gradient PCR system, Eppendorf AG, Germany) was programmed to first incubate the sample for 5 minutes for 95℃ followed by 45 cycles consisting of 94℃ for 45 seconds, 55℃ for 50 seconds and 72℃ for 1 minute, 30 seconds respectively with final extension for 8 minutes at 72℃. The PCR amplified products were identified by gel electrophoresis.

At the end of the run the gel was under transilluminator gel doc and bands of amplified product were evaluated. The amplified product was compared with 100-bp DNA ladder (GibcoBRL, Life Technologies) and mutation was detected and compared with control positive and negative cases.

Point mutations located at positions 12 in exon 1 were dectected. PCR primers were used for amplification. *KRAS* positive control was used by repeating the positive cases which were detected during this project and for negative control water was used.

2.1 Data Analysis

Data was entered on a data sheet. Percentage of KRAS positive mutations in neoplastic and non neoplastic lesions were calculated.

3. RESULTS

KRAS mutations located at codon 12 exon 1 were found in 02(2.87%) cases of multinodular goiter while 05(7.14%) hyperplastic nodules were positive for KRAS. In benign neoplastic lesions of thyroid 02 (2.87%) follicular adenoma showed KRAS mutations. Among borderline lesions (WDT-UMP) 03(4.2%) cases were KRAS positive. Among malignant lesions most of follicular carcinoma showed KRAS positivity 03(4.2%). Amongst 22 classical papillary carcinoma only 08(11.42%) showed KRAS mutations whereas out of 11 cases of follicular variant of papillary carcinoma 07(10%) showed KRAS mutations.

4. DISCUSSION

The thyroid nodular lesions have marked heterogeneity varying from multinodular goiters, adenomas to carcinomas. The histopathological diagnosis of thyroid lesions has been a significant problem and has been a subject of debate in the literature [17].

Our study showed mutated KRAS at codon 12 on exon 1, showing conformity with other studies also reported similar findings. A higher proportion of positive KRAS was found in neoplastic lesion 23 (32.69%) followed by non-neoplastic 7(10.1%). Our findings are comparable to international studies who also reported that KRAS mutations are more frequent in neoplastic lesions than in non-neoplastic lesions [8].

4.1 KRAS in Benign Lesions

Amongst the benign lesions in this study *KRAS* mutations were present in 2.87% multinodular goiter. The frequency of *KRAS* mutations in multinodular goiter varies in different studies ranging from 0% to 10%, 2.17% and 1% respectively [8,18]. Overall RAS mutations i.e. NRAS, KRAS and HRAS are reported as

Table 1. Distribution of selected neoplastic and non-neoplastic lesions of thyroid according to KRAS mutations (n=70)

Morhological type	KRAS mutations +ve (%)	KRAS mutations –ve (%)
Neoplastic		
FA	2 (2.87%)	8 (11.42%)
WDTUMP	3 (4.2%)	4 (5.71%)
FC	3 (4.2%)	1 (1.4%)
PTC	8 (11.42%)	14 (20%)
FVPTC	7 (10%)	4 (5.71%)
Total	23(32.69%)	31(44.24%)
Non-neoplastic		
MNG	2 (2.87%)	4 (5.71%)
HN	5 (7.14%)	5 (7.14%)
Total	7(10.1%)	9(12.85%)

FA = Follicular adenoma, WDUMP = Well differentiated tumor of uncertain potential, PT = Papillary thyroid carcinoma, FVPTC = Follicular variant of papillary carcinoma, MN = Multinodular goiter

0%, 6.5% and 7%. Our study observed KRAS mutations in a small percentage of hyperplastic nodules i.e 7.14%. The overall reported RAS mutations in hyperplastic nodules are 5.6% [18]. These benign lesions may later transform into neoplastic lesions and further support the hypothesis of Nikiforov [10] that nodules harbouring KRAS mutations may be categorized as follicular adenoma as they are probable true neoplasms.

Regarding benign neoplastic lesions, KRAS mutations were found in 2.87% cases of follicular adenoma. Our findings are comparable to Vasko et al. [8] reporting exon 1 KRAS mutation in 0.6% of follicular adenoma while most of their cases showed NRAS mutations i.e 14%. Our results could not be compared to other studies because studies regarding KRAS mutations in follicular adenomas are limited.

4.2 KRAS in Malignant Lesions

Follicular carcinoma in this study shows 4.2% KRAS mutation. Our results are slightly higher as compared to Vasko et al. [8] who reported exon 1 KRAS mutations in 1.6% cases of follicular carcinoma while most of their cases showed NRAS mutations. Nikiforov et al. (2003) and Schulten et al. (2013) on the contrary reported that none of their cases of follicular carcinoma showed KRAS mutation [18,19]. Nikiforov [10] has reported overall RAS mutations in 40% to 50% of conventional type follicular carcinoma, however no specific RAS isoform was mentioned in his study. Garcia-Rostan et al. [16] have reported correlation between RAS mutations and metastatic behavior of tumor. Our figures could be related to tumor aggressiveness in KRAS positive follicular carcinoma. Further studies are required on extensive sample size for detection of KRAS mutations in follicular carcinoma.

For follicular variant of papillary thyroid carcinoma this study showed mutated KRAS in codon 12 on exon 1 in 10% cases. Our results are higher in comparison with Rivera et al. [20] who reported KRAS mutations in a small number of cases 1/28 (3.57%) and Park et al. [21] reports 22.9%. Zhu et al. [22] on the contrary reported that none of his cases of FVPTC showed KRAS mutation. According to Park et al. [21] KRAS mutations were positive in most papillary carcinoma with follicular growth pattern suggesting phenotypic difference between classical papillary carcinoma and its follicular variant. According to Rivera et al. [20] follicular variant of papillary carcinoma has a molecular profile close to follicular adenoma/carcinoma. Nikiforov [10] has postulated that KRAS positive papillary carcinoma with follicular histology show frequent encapsulation, less obvious nuclear features of papillary carcinoma and lower chance of lymphatic spread. The present study however, emphasizes the association of FVPTC with KRAS mutations in our population.

Classical papillary carcinoma in this study showed 11.4% positivity for KRAS mutations. These figures are higher compared to international study reporting 2.7%. A higher percentage of 54.5% was reported by Schulten et al. [18] Overall RAS i.e. KRAS, NRAS & HRAS mutations are reported by Esapa et al. (1999) 8%, Basolo et al. (2000) 10% and Zhu et al. (2003) 17% respectively [22,23,24]. Ahigher result was expected in our study but according to Myers et al. (2012) and Vasko et al. (2003) discrepancy may be due to small sample size, epidemiological differences and as well as due to

the fact that though KRAS mutations are often present in higher numbers of tumors, but exists as mutant fraction that are too small to be detectable by direct sequencing that can be missed by Real time PCR and can only be detected by ACB-PCR (Allele –specific competitive blocker PCR) [3,8].

Present study showed KRAS mutations (4.2%) in well differentiated tumors of uncertain malignant potential (WDT-UMP) According to Hofman et al. [25] none of his cases showed KRAS mutations while NRAS was present in 1/31(3.22%) cases. Probably these figures are related to likely hood of a possible malignant transformation in these lesions. The information regarding KRAS mutation in WDT-UMP was limited in literature so comparisons were difficult.

The presence of oncogenic KRAS is a marker of tumor aggressiveness and poor outcome and may guide future course of treatment.

5. CONCLUSION

Majority of malignant tumors showed KRAS mutations supporting the presence of KRAS mutations in our population. Follicular variant is a discrete variant of papillary carcinoma having strong association with KRAS mutations whereas Classical papillary carcinoma did not showed significant KRAS mutations as mutated KRAS are often present as mutant fraction which can be missed by DNA sequencing techniques, even by quantitative real time PCR and can only be detected by more sensitive techniques such as ACB-PCR (Allele –specific competitive blocker PCR).

Benign lesions harbouring KRAS mutations may serve as malignant precursors. This study showed that KRAS mutation were found in variable percentages of various thyroid proliferative lesions ranging from multinodular goiters, hyperplastic nodules, follicular adenomas, WDT-UMP, classical papillary carcinomas, and follicular variant of papillary carcinomas.

6. RECOMMENDATIONS

1. More studies on other centers of Pakistan are needed to know the actual prevalence of KRAS.
2. Malignant tumors particularly classical papillary carcinoma should be evaluated for KRAS mutations as tumors carrying KRAS mutations are more aggressive and unlikely to benefit from targeted therapies such as Epidermal growth factor receptor (EGFR) as KRAS leads to its downregulation.

7. LIMITATION

Due to financial constraints standard PCR was done and only 70 samples were tested.

Secondly patient compliance was low therefore follow up of patients were not possible.

Hospital records were not available for correlation of staging of tumors with mutations.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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